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Erythropoietin and Mild Traumatic Brain Injury: Neuroprotective Potential and Dangerous Side-effects

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Abstract: The aim of this study was to assess the neuroprotective effects of recombinant human erythropoietin relating to mild traumatic brain injuries. Secondary stressors following mild traumatic brain injury (mTBI) are a significant source of neuronal damage. However, the temporal delay in the onset of these secondary mechanisms allows for potentially therapeutic interventions. Recombinant human erythropoietin (r-Hu-EPO) has demonstrated neuroprotective effects when administered post-injury, but neuronal survival following chronic pre-injury administration of r-Hu-EPO has yet to be described. In the present study, female Wistar rats receiving mTBI were administered r-Hu-EPO in one of two different treatment regimens. The first was a strictly therapeutic dose, administered after the injury. The second was a multi-dose regimen designed to replicate the effects of chronic r-Hu-EPO use. Control subjects received physiological saline. Morris water maze (MWM) testing revealed no significant differences between experimental groups. Contextual fear paradigms showed that the normal extinction of the fear response was absent in mTBI animals, regardless of r-Hu-Epo treatment. Histological analyses indicated that both therapeutic and chronic administration of r-Hu-EPO resulted in increased cortical neuron survival following mTBI. However, numerous side effects associated with chronic r-Hu-EPO use, but not the therapeutic dose, were noted following mTBI. These included post-injury seizure, cerebral hematoma and intracerebral hæmorrhage, among others. These findings identify a significant health risk for individuals engaging in chronic r-Hu-EPO misuse, as is the case in the large sub-population of amateur and professional athletes taking r-Hu-EPO for its performance enhancing properties.

Key words: Erythropoietin, TBI, neuroprotection, conditioned fear

INTRODUCTION

Estimates based primarily on hospital records indicate that approximately 1.5 million persons in the United States sustain a brain injury annually (Rutland-Brown *et al*., 2006; Sosin *et al*., 1996). While a vast majority of the cases requiring hospitalization are mild traumatic brain injuries (mTBIs) (Miller, 1993), many more mTBIs often go unreported and are not accurately represented in these estimates (Bohnen and Jolles, 1992; Coonley-Hoganson *et al*., 1984). Sosin *et al*. (1996) suggest that mTBIs occur at a rate of 618/100000, with sport-related injuries being a notable contributor. Other researchers have found a comparable frequency of mTBI in the US (Bazarian *et al*., 2005; Rutland-Brown *et al*., 2006) and elsewhere (Wrightson and Gronwall, 1998). Invariably, such mild head traumas result in some degree of cortical neuron damage and often produce protracted cognitive, emotive and social deficits in the injured patient.

While the initial traumatic insult usually generates immediate, irreversible brain damage, a significant amount of secondary neuronal damage also occurs in the hours and days following the primary injury. This secondary damage has been associated with a number of mechanisms, including inflammation (Brines *et al*., 2000; Sakanaka *et al*., 1998) glutamate toxicity (Bernaudin *et al*., 1999; Morishita *et al*., 1997; Sakanaka *et al*., 1998) (associated with excessive Ca^{+2} influx), secondary ischæmia or hypoxia (Ribatti *et al*., 1999), free radical production (Boran *et al*., 1998; Cavdar *et al*., 1997) and delayed apoptosis (Du *et al*., 1996). However, due to their temporal profiles, these secondary mechanisms have been identified as potential therapeutic targets. Many pharmacological agents are now being tested as possible means of attenuating secondary brain damage following mTBI.

Erythropoietin (EPO) is a naturally occurring glycoprotein that stimulates the proliferation and differentiation of erythroid progenitor cells (Fisher, 1997;

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Graber and Krantz, 1978). Recombinant human EPO (r-Hu-EPO) is synthesized in the laboratory for clinical and experimental use and is biologically active in a number of mammals (Egrie *et al*., 1988; Sasaki *et al*., 2001). Originally intended as a treatment for anemia, r-Hu-EPO has gained popularity in recent years as an experimental neuroprotective agent, as it has demonstrated an efficacy for attenuating cortical neuron damage following neuronal insults of varying etiologies (Bernaudin *et al*., 1999; Brines *et al*., 2000; Marti, 2004; Siren *et al*., 2001; Wang *et al*., 2004a, b). A duality also exists in r-Hu-EPO use, in that it is a commonly abused substance in amateur and professional sport (Gareau *et al*., 1996; Jelkmann, 2000; Sawka *et al*., 1996; Spalding, 1991; Spivak, 2001). Chronic r-Hu-EPO use in the healthy patient results in an increase in erythrocytes, which improves the oxygen carrying capacity (OCC) of the blood. Studies have shown an improvement in lung oxygen capacity (Berglund and Ekblom, 1991; Ekblom *et al*., 1972) and delayed exhaustion when training on r-Hu-EPO (Ekblom *et al*., 1972). An interesting paradigm thus arises in contact sports where repeated head impacts are frequent and where EPO may be introduced into the system for 'doping' purposes prior to a cortical impact.

The intent of this study was twofold: to assess the neuroprotective effect of EPO when administered post-injury and to identify the potential benefits and/or hazards of EPO misuse in the healthy patient who is prone to repeated mTBI's (i.e., in sport). Behavioural measures of spatial and contextual memory were analyzed as indicators of cognitive functioning and post-injury cortical neuron viability was quantified histologically.

MATERIALS AND METHODS

Subjects: Twenty-four adult female Wistar albino rats (Charles River, QC) served as subjects. Rats were housed in mesh-bottomed cages in groups of two or three. A 12:12 hour light:dark cycle was used. Room temperature was maintained at 21±1°C and food and water were available ad libitum. Animals' body weights were measured daily. All experimental procedures were approved by the Laurentian University institutional Animal Care Committee and complied with the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals. All procedures were completed in the Paul Field animal care facility during the 2006 calendar year.

Mild Traumatic Brain Injury (mTBI): Methods for mTBI application (mechanical impact model) have been described previously by our laboratory (Lado and Persinger, 2003). Rats were returned to their home cages after normal ambulatory behaviour had resumed and were closely monitored in the following hours for any conspicuous health concerns. Sham-injured animals were exposed to the same handling conditions with the exception of injury being inflicted.

R-Hu-EPO Administration: EPREX® epoetin alfa obtained from Ortho Biotech (Janssen-Ortho Inc., Toronto, ON) was administered intraperitoneally (i.p.) at a dose of 2000 IU kg^{-1}. Rats received a total of either two or eight doses, based on the following two injection regimes: I) 2 injections (at 3 and 24 h post-mTBI) or II) 6 injections (once every 48 h for twelve days) prior to mTBI plus 2 injections at 3 and 24 h post-mTBI. The former was intended strictly as a therapeutic intervention following mTBI while the latter was designed to stimulate the hæmatopoietic response induced in chronic EPO abuse. These injection regimens are herein referred to as the therapeutic and chronic administration of r-Hu-EPO, respectively. Control subjects received an equal volumetric dose of physiologic saline (1 mL kg^{-1}).

Behavioural testing

Morris water maze: Morris Water Maze (MWM) tests were completed for 3 consecutive days prior to and 3 consecutive days following application of mTBI. Each subject completed four trials per day for a total of 12 pre-injury and 12 post-injury trials. MWM consisted of a 1.5 m-diameter pool, arbitrarily divided into quadrants and filled with approximately 20 cm of water. A non-visible platform was fixed in one quadrant 2 cm below water level and 25 cm from the pool wall. On each trial, rats were placed into the water by hand, facing the wall. Starting points were assigned randomly but did not include the quadrant in which the platform was located. Distal visual cues were fixed throughout the room for the duration of the experiment. Escape latency (in seconds), time spent in each quadrant and thigmotaxic behaviours were recorded for each subject. Completion of a trial involved the rat locating the platform and remaining there for 30 sec. A maximum of 120 sec was allotted per trial, after which time the rat was guided to the platform and remained there for 30 sec. Water temperature was maintained at 24 ±1°C.

Conditioned fear testing: Subjects were placed in a conditioning chamber for 6 min and after an initial 3 min habituation period, were exposed to three unsignalled foot shocks (2 mA, 2 sec duration) at t = 3, 4 and 5 min, respectively. Subjects were then returned to the chamber for 8 min observation periods (no shocks) at 24, 48, 96, 120 and 144 h after the initial exposure (mTBI occurred at 72 h). Locomotor activity was recorded based on whether or not the subject was moving during 8 sec intervals. A

score of 1 denoted movement while a score of 0 denoted no movement. The total score for motility thus ranged from 0 to 60 and was a quantification of the typical freezing response encountered in fear-conditioned rats (Bouton and Bolles, 1979; Fanselow and Bolles, 1979). A similar paradigm was repeated at 10 days post-injury, with another conditioning day (3 unsignalled shocks) followed by four consecutive observation days. Extinction co-efficients were generated for the learned freezing response measured in each paradigm. All behavioural testing was counterbalanced.

Preparation of brain tissue and histological analysis: Following CO euthanization and decapitation, brains were rapidly extracted and fixed in ethanol-formalin-acetic acid. Brains were later embedded in paraffin and 10 μm coronal sections were obtained. Sections were stained with Toluidine Blue O (Nissl stain) and assessed under light microscopy at 400X magnification for the presence of anomalous neurons in cortical and counter-coup regions. Anomalous neurons were characterized by nuclear pyknosis, shrunken elongate soma and excessive uptake of stain. Coronal sections were analysed by quadrants, with the midline dividing right and left hemispheres and the rhinal fissure being the demarcation between upper and lower quadrants. The degree of cortical neuron damage was quantified as the number of anomalous neurons per unit area. Damage scores were then computed for hemisphere and whole coronal sections. All subjects were allowed to recover at least four full weeks after injury before brain tissues were collected, with the exception of subjects used for hematocrit analysis.

Evaluation of hematocrit: Whole blood was taken from a sample of subjects at time of death for verification of hæmatopoietic response to chronic r-Hu-EPO administration. Blood was centrifuged for 5 min at 2000 rpm. Hematocrit was then measured manually using a standard scale on a microhematocrit reader. Subjects used for hematocrit measurement were harvested within two-weeks of final r-Hu-EPO injection.

RESULTS

Changes in body weight following mTBI: Rats receiving mTBI displayed a conspicuous decline in mean body weight following injury ($F(1, 22) = 6.30$, $p<0.024$, $eta^2 = 0.27$) that was not evident in sham-injured animals (Fig. 1). Approximately 7-8% of pre-injury body weight was lost following mTBI and was still not regained four weeks later when measurements had concluded. This protracted weight loss has been described previously by Lado and Persinger (2003).

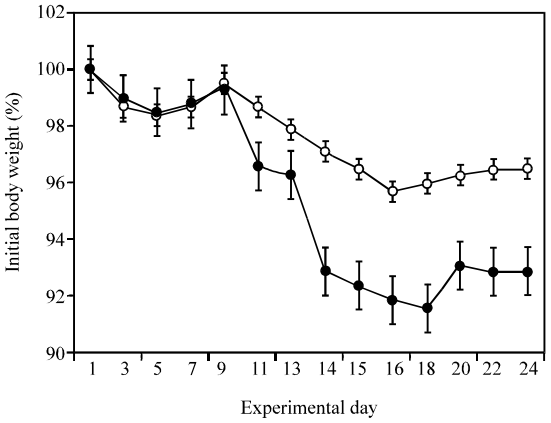

Fig. 1: Percent change from initial body weight. Animals receiving mTBI (closed circles) showed a marked weight loss in the days following the injury. The weight was not yet regained when measurements had concluded 4 weeks post-injury. SHAM animals (open circles) did not display this effect. The mTBI occurred on experimental day 13. Error bars represent standard error of the means

Behavioural measures

Morris water maze: Mild TBI in rats did not result in any significant impairment in Morris Water Maze (MWM) performance; this finding was independent of r-Hu-EPO treatment conditions. All subjects showed an improvement in escape latencies over days of testing ($F_{(5,70)} = 8.45$, $p<0.01$, $eta^2 = 0.38$) indicating a learning of the spatial task over time. On any given day, there were no differences between treatment conditions regarding escape latency, thigmotaxic behaviour, or time spent in the quadrant where the platform was located (all p's>0.05).

Fear conditioning: Two fear-conditioning paradigms were used in the present study. The first was initiated prior to the application of mTBI with subsequent observation days continuing into the pre- and post-injury period. The second paradigm was initiated after the injury, with the pairing day occurring 10 days post-mTBI. With respect to the first paradigm, all groups showed a net tendency to increase movement with each passing day of observation ($F_{(8,96)} = 10.91$, $p<0.01$, $eta^2 = 0.48$). This may be regarded as a normal extinction of the fear response. Initial analyses indicated no difference in mean freezing scores between mTBI and sham-injured rats (p>0.05). Moreover, the rate of extinction of the fear response was similar for mTBI and sham-injured animals during the first exposure to the fear chamber. In the second fear conditioning paradigm, similar results were recorded with respect to mean freezing scores for mTBI and sham-injured animals. However, mTBI-

treated rats failed to exhibit an extinction of the learned freezing response during the second fear conditioning paradigm ($t(13) = 2.56$, $p = 0.024$) while sham-injured animals showed consistent extinction rates between paradigms ($p>0.05$).

Histological analyses: Analysis of variance indicated that the experimental mTBI did produce significant neuronal damage ($F_{(1,22)} = 4.73$, $p<0.05$, $eta^2 = 0.23$). However, post-hoc tests indicate that only mTBI rats given saline display a high degree of neuronal death compared to sham-injured animals ($F_{(1,10)} = 9.17$, $p = 0.014$, $eta^2 = 0.50$), while mTBI rats receiving r-Hu-EPO treatment do not ($p>0.05$). This neuroprotective property of r-Hu-EPO was independent of injection regimen. To analyze the spatial distribution of neuronal damage, coronal sections were divided vertically (at the midline) and horizontally (at the level of the rhinal fissure) to produce four quadrants. In the saline treated mTBI animals, diffuse neuronal damage was noted in each of the quadrants, with no quadrant displaying more extensive damage than any other (all p's>0.05). The impact occurred at the dorsal surface of the upper right quadrant. Figure 2 is a micropictograph showing examples of cortical neurons following sham-injury and mTBI with either saline, therapeutic r-Hu-EPO administration, or chronic r-Hu-EPO administration (Panels A, B, C and D, respectively). Administration of r-Hu-EPO in sham-injured animals did not influence cortical neuron viability ($p>0.05$).

Hematocrit values: Analyses of blood hematocrit levels were conducted in a sub-population of rats ($n = 8$). A two-way analysis of variance comparing mean hematocrit counts by drug condition demonstrated a significant increase ($F_{(1,9)} = 50.75$, $p<0.02$, $eta^2 = 0.67$) in hematocrit levels for the groups receiving r-Hu-EPO injections compared to saline-treated control subjects. Both the chronic and the therapeutic injection regimens elicited a hematopoietic response and the magnitude of the response was comparable for each ($p>0.05$).

Health risks associated with r-Hu-EPO administration: A number of health risks associated with the administration of r-Hu-EPO were noted during the course of this experiment. Most of the associated risks occur when r-Hu-EPO is chronically administered and concomitant with mTBI. The primary areas of concern include one fatality, which was observed following mTBI. Post-mortem findings suggest that cause of death was related to a large cerebral hematoma on the right lateral surface of the caudal frontal lobe (Fig. 3). The same animal presented a conspicuous intracerebral hemorrhage, bordered medially by the forceps major of the corpus

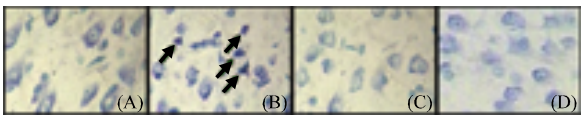

Fig. 2: Cortical Neuron Morphology Following Brain Injury. When assessed under light microscopy at 400X magnification, the cortical neurons of sham-treated brains retained their typical size and pyramidal shape (Panel A). Following mTBI, damaged cortical neurons were shrunken and more densely stained (arrows); there is also evidence that the nuclear membrane was compromised (Panel B). When mTBI was concomitant with either the chronic or therapeutic administration of r-Hu-EPO, cortical neurons appeared normal (Panels C and D, respectively)

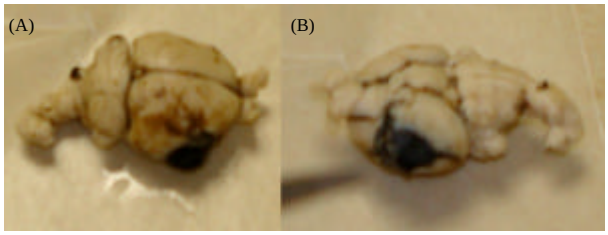

Fig. 3: Cerebral Hematoma following mTBI. One rat receiving the chronic administration of r-Hu-EPO exhibited a hematoma following mTBI. The hematoma, shown above from dorsolateral (A) and ventrolateral (B) views was located on the lateral surface of the caudal right frontal lobe. The site of impact was the dorsal right parietal area

callosum, during histological observation (Fig. 4). This animal had received the chronic administration of r-Hu-EPO prior to application of mTBI.

A two-way analysis of variance for the amount of time required to recover from the mechanical impact injury (i.e., stun time) was conducted for all experimental groups receiving the mTBI treatment. A significant increase in stun times ($F_{(3,17)} = 2.15$, $p<0.05$, $eta^2 = 0.37$) was detected for mTBI animals receiving the chronic administration of r-Hu-EPO compared to saline-treated mTBI animals. No stunning was observed in animals given the sham-injury.

Post-injury seizures were noted immediately following mTBI in two of the four rats that received chronic r-Hu-EPO administration with the injury. Seizures were characterized by bilateral paresis (originating on the left-side) following forelimb clonus, rearing, loss of balance and falling. No other experimental groups exhibited post-injury seizures.

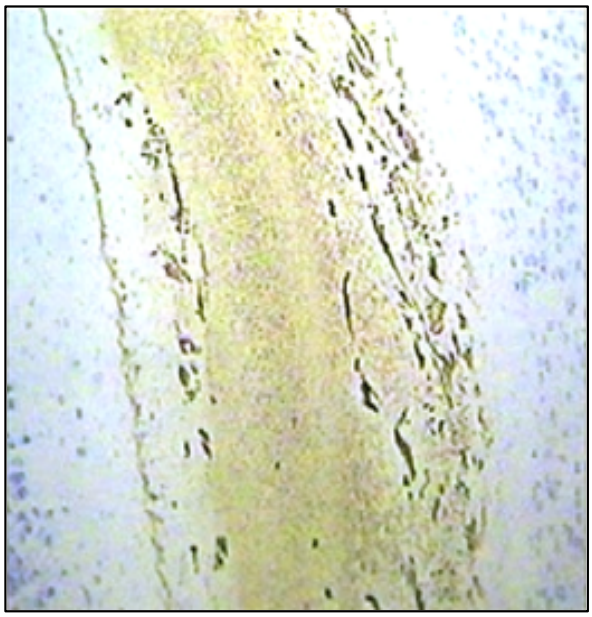

Fig. 4: Intracerebral Hemorrhage. One animal receiving the chronic administration of r-Hu-EPO showed extensive intracerebral haemorrhaging (ICH) following mTBI. This was the same animal exhibiting the cerebral hematoma. At its greatest extent, the blood tissue was bordered medially by the forceps major of the corpus callosum and extended to the lateral surface of the right hemisphere

Additional post-mortem analyses revealed an enlarged spleen in one of the rats chronically treated with r-Hu-EPO. The approximately 5.1 g spleen was significantly larger than usual and a large encapsulation, which visually resembled a splenic hematoma, occupied the distal quarter of the organ.

DISCUSSION

There are multiple references in the literature to body weight loss following brain injury. This process is reported to follow various etiological sources of brain injury, including trauma from mechanical impact (Lado and Persinger, 2003; Lyeth *et al*., 1992) and ischemia (Lei *et al*., 2001). In some circumstances, pharmacologic intervention can attenuate this body weight effect (Kumon *et al*., 2000; Lei *et al*., 2001; Lyeth *et al*., 1992); however few theories regarding the mechanism(s) of action are put forward.

The current study failed to replicate previous findings of brain injury causing deficits in MWM performance (Kumral *et al*., 2004; Lu *et al*., 2005). This may be due in part to the etiological variation between studies (i.e., ischemic vs. traumatic insults) or the severity of the mechanical trauma. Additionally, there was an intentional allowance for a small degree of variability regarding the site of impact. However, such latitude in the specificity of impact location was required to create a generalizable model that replicates mTBI in the human population. Consequently, MWM testing alone may not be sensitive enough to detect the behavioural sequelae associated with the proportion and distribution of cortical neuron damage reported in our study. It is evident that multiple behavioural measures should be employed to optimally detect any deficits associated with very mild brain injuries. Alternatively, there may be a critical number of neurons required for the execution of this particular function. If the amount of damage produced by the mTBI does not intrude upon this critical mass, then certain behavioural manifestations noted in more severe injuries will not arise here.

In a contextual learning paradigm, it is normal for the learned response to extinguish over time if the aversive stimulus is not reinforced. Within our conditioned fear experiments, we did observe such an extinction of the fear response (freezing) as days progressed, but only if the animal was paired with the aversive stimulus prior to the injury. That the rats exposed to the identical aversive context after mTBI maintained the freezing response in the absence of reinforcement suggests an inability to adapt to environmental conditions. It is noteworthy in itself that the mTBI-injured animals appeared to respond appropriately to the aversive stimulus on the first day of observation, despite the lack of extinction.

Present findings regarding an elevation of hematocrit suggests that r-Hu-EPO may elicit a haematopoietic response with a relatively lower dose than what is often reported (Brines *et al*., 2000; Kato *et al*., 1998). We found no difference in hematocrit between chronically administered (8 doses at 2000 IU kg^{-1} over 14 days) and therapeutically administered (2 doses at 2000 IU kg^{-1} over 2 days) r-Hu-EPO. Whole blood samples were taken for analysis up to three weeks after the cessation of treatment, indicating that even acute r-Hu-EPO administration can cause substantial and protracted elevations in erythrocyte levels. If increased blood viscosity or other hematocrit-related parameters are responsible for some of the health hazards reported here, then users should be aware of the duration and magnitude of the haematopoietic response.

Our histological data demonstrating the neuroprotective effects of r-Hu-EPO is consistent with the existing literature (Matchett *et al*., 2006; Sadamoto *et al*., 1998; Siren *et al*., 2001, 2006). There are numerous mechanisms by which r-Hu-EPO is proposed to exert its neuroprotective effect, including anti-oxidant or

angiogenic properties of the drug as well as biochemical cascades initiated by stimulation of the EPO receptor (Brines *et al*., 2000; Digicaylioglu and Lipton, 2001; Marti *et al*., 1996; Sakanaka *et al*., 1998). Almost invariably, it is implied that r-Hu-EPO be present in the bloodstream (or cerebrospinal fluid) to exert its neuroprotective effects. It may be possible that an increased hematocrit at the time of injury could confer a neuroprotective advantage without having circulating levels of r-Hu-EPO. In such a case, the increased oxygen carrying capacity of the blood could be sufficient to compensate for some of the oxidative stress placed on the neuronal environment during very mild head injuries. The timeframe and dosing of our chronically treated r-Hu-EPO rats was specifically chosen to ensure that negligible amounts of the drug (<1%) (Egrie *et al*., 1988; Kaufman *et al*., 1998) were in circulation at the time of the injury. However, since no additional neuronal sparing was observed in the chronically treated r-Hu-EPO groups following mTBI, the authors suggest that it is primarily the presence of circulating r-Hu-EPO and not the increase in erythrocytes, that contributes to the neuroprotective effects seen here.

Present findings of an intracerebral hemorrhage and large cerebral hematoma following such a mild brain injury are by far the most troubling. One of four animals receiving chronic r-Hu-EPO with concomitant mTBI displayed these symptoms. It is possible that r-Hu-EPO use may increase the severity of haemorrhaging in the normal patient because of its predisposition for clot formation (Murphy and Parfrey, 1999). A systemic increase in clot formation will somewhat deplete the amount of circulating clotting factors (Cooper and Cooper, 1977), causing an injury to result in more substantial bleeding than usual. However, numerous other factors must be considered, including any angiogenic and vasoactive properties of r-Hu-EPO, as well as its effect on blood viscosity. Moreover, two of four rats receiving the chronic administration of r-Hu-EPO displayed immediate-onset post-traumatic seizures. While such seizures have been reported, this phenomenon typically occurs in less than five percent of head injured individuals (Annegers *et al*., 1980; Moon *et al*., 1999; Sander *et al*., 1990) and is more common with moderate or severe brain injuries (Annegers *et al*., 1998). In addition, our findings of a significantly increased stun time in mTBI rats following injury may be analogous to the partial seizures described by Ryan *et al*. (1997). following mild head injury. When taken collectively, the hemorrhage/hematoma, seizures and increased stun times provide convincing evidence that chronic administration of r-Hu-EPO prior to a mild mechanical injury to the head has highly damaging, if not lethal consequences.

CONCLUSIONS

The ability of r-Hu-EPO to be neuroprotective in a wide range of neuronal populations following a variety of etiologies demonstrates its continued importance as an experimental therapeutic intervention for brain injury. While the experimental use of r-Hu-EPO as a treatment for mild traumatic brain injury is not novel, we are the first to report the above described side effects of chronic r-Hu-EPO administration associated with mTBI. We believe that the identification of these health hazards poses serious implications for the large population of amateur and professional athletes chronically misusing r-Hu-EPO (Bento *et al*., 2003; Magnani *et al*., 1999). This is particularly true for athletes engaging in sports where head injuries are frequent and often repetitive.

Authors Contributions: Each section of the study was completed by P Evans and M A Persinger in the percentages of 85 and 15, respectively. Procedures included are experimental design, testing, data analysis and manuscript preparation.

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